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Clostridium innocuum, an emerging pathogen that induces lipid raft-mediated cytotoxicity

Hui-Yu Wu[a*], Chia-Jung Kuo[b,c,d*], Chia-Huei Chou[e,f*], Mao-Wang Ho[e,f], Chyi-Liang Chen[a,g], Tsui-Shan Hsu[a], Ying-Chu Chen[a], Chuan Chiang-Ni[a,g], Yi-Ywan M. Chen[a,g], Cheng-Hsun Chiu[a,b,c,g], and Chih-Ho Lai [a,f,g,h]

[a]Department of Microbiology and Immunology, Graduate Institute of Biomedical Sciences, Chang Gung University, Taoyuan, Taiwan; [b]School of Medicine, College of Medicine, Chang Gung University, Taoyuan, Taiwan; [c]Chang Gung Microbiota Therapy Center, Chang Gung Memorial Hospital at Linkou, Taoyuan, Taiwan; [d]Department of Gastroenterology and Hepatology, Chang Gung Memorial Hospital at Linkou, Taoyuan, Taiwan; [e]Department of Infectious Disease, China Medical University Hospital, Taichung, Taiwan; [f]Department of Infectious Disease, Department of Microbiology and Immunology, School of Medicine, China Medical University, Taichung, Taiwan; [g]Molecular Infectious Disease Research Center, Department of Pediatrics, Chang Gung Memorial Hospital at Linkou, Taoyuan, Taiwan; [h]Department of Nursing, Asia University, Taichung, Taiwan

ABSTRACT

Clostridium innocuum is an emerging spore-forming anaerobe that is often observed in *Clostridioides difficile*-associated inflammatory bowel disease (IBD) exacerbations. Unlike *C. difficile*, *C. innocuum* neither produces toxins nor possesses toxin-encoding genetic loci, but is commonly found in both intestinal and extra-intestinal infections. Membrane lipid rafts are composed of dynamic assemblies of cholesterol and sphingolipids, allowing bacteria to gain access to cells. However, the direct interaction between *C. innocuum* and lipid rafts that confers bacteria the ability to disrupt the intestinal barrier and induce pathogenesis remains unclear. In this study, we investigated the associations among nucleotide-binding oligomerization domain containing 2 (NOD2), lipid rafts, and cytotoxicity in *C. innocuum*-infected gut epithelial cells. Our results revealed that lipid rafts were involved in *C. innocuum*-induced NOD2 expression and nuclear factor (NF)-κB activation, triggering an inflammatory response. Reducing cholesterol by simvastatin significantly dampened *C. innocuum*-induced cell death, indicating that the *C. innocuum*-induced pathogenicity of cells was lipid raft-dependent. These results demonstrate that NOD2 mobilization into membrane rafts in response to *C. innocuum*-induced cytotoxicity results in aggravated pathogenicity.

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KEYWORDS
Clostridium innocuum; cholesterol; lipid rafts; cytotoxicity; inflammation

Introduction

Clostridium innocuum, a spore-forming Gram-positive anaerobe, is a crucial pathogen for extraintestinal clostridial infections (EICIs) [1], such as peritonitis [2], bacteraemia [3], and endocarditis [4]. We recently reported that *C. innocuum* is the second most common pathogen causing EICIs, with a higher mortality rate than other clostridial species [5]. Vancomycin-resistant *C. innocuum* is found in patients with recurrent *C. difficile*-associated diarrhoea [6]. The incidence of *C. innocuum* infection in hospitalized patients with inflammatory bowel disease (IBD) is higher than that of *C. difficile* infection [7]. Notably, the severity of the disease is higher in patients infected with *C. innocuum* than those infected with *C. difficile* [8], indicating that *C. innocuum* is a bona-fide pathogen that causes gastrointestinal and extraintestinal infections.

The virulence factors of *C. difficile* include germination and outgrowth of spores and two glucosylating toxins (toxin A and toxin B) [9]. Cytotoxins produced by *C. difficile* play a crucial role in intestinal pathogenesis and are associated with severe colitis [10]. Unlike *C. difficile*, *C. innocuum* does not contain any genes encoding cytotoxins, and the bacterial pellets rather than the culture supernatant indeed exhibit cytotoxicity in human colon epithelial cells [11].

Nucleotide-binding oligomerization domain containing 2 (NOD2), a human membrane-associated innate immune receptor, is abundantly expressed in the gut epithelium and sensitizes muramyl dipeptide (MDP) in the bacterial cell wall [12]. Activation of NOD2 induces receptor-interacting protein 2 (RIP2) recruitment, which then initiates nuclear factor (NF)-κB signalling to produce inflammatory cytokines [13]. *C. innocuum* contains cell wall proteins, similar to *C. difficile* surface layer proteins, causing cytotoxicity [11]. However, whether NOD2 is involved in *C. innocuum*-induced pathogenesis remains unknown and requires further investigation.

CONTACT Cheng-Hsun Chiu chchiu@adm.cgmh.org.tw; Chih-HoLai chlai@mail.cgu.edu.tw
*Equal contribution to this work.

Lipid rafts are membrane microdomains composed primarily of dynamic assemblies of sphingolipids and cholesterol [14]. Several pathogens exploit lipid rafts for initial attachment to the cell membrane and gain access to the host cells [15–18]. *C. innocuum* can be predominantly identified from a bacterial consortium in the creeping fat of IBD ileal surgical resections [19]. Destruction of the intestinal barrier enhances *C. innocuum* translocation from the gut lumen across the barrier and tropism to the mesenteric adipose tissue, implying a preference for lipid-rich environments. However, whether the direct interaction between *C. innocuum* and lipid rafts confers the bacteria the ability to disrupt the intestinal barrier and induce pathogenesis needs to be investigated. In this study, we explored the molecular mechanisms of *C. innocuum*-induced pathogenesis in colonic epithelial cells. We further investigated whether NOD2 mobilized to membrane rafts was implicated in *C. innocuum*-induced cytotoxicity.

Materials and methods

Bacterial culture

C. innocuum was cultured on CDC anaerobe agar containing 5% sheep blood (BD, Franklin Lake, NJ, USA) under anaerobic condition for 2 days at 37°C. *C. innocuum* was inoculated in Brain-Heart Infusion broth supplement with 0.5% yeast extract and 0.1% L-cysteine as described previously [5].

Cell culture

HT-29 cells (ATCC HTB-38) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% foetal bovine serum (FBS) and incubated at 37°C with 5% CO_2. For simvastatin treatment, HT-29 cells were cultured in DMEM containing 2% FBS.

Western blot assay

HT-29 cells were infected with *C. innocuum* at different multiplicity of infections (MOI) for different times. Cell lysates (60 μg) were loaded in 7.5% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) for 90 minutes. The membranes were blocked with Tris-buffered saline with Tween 20 (TBST) containing 5% skim milk for 1 h at room temperature. The membranes were then incubated with primary antibodies against NOD2 (GTX306615, dilution 1: 200), RIP2 (CST4142, dilution 1:1000), and β-actin (dilution 1:5000) at 4°C overnight. Th membranes were then incubated with horseradish peroxidase (HRP)-conjugated antibody for 1 h. The proteins of interest were detected with ECL Western Blotting Detection Reagent (BIOMAN, Taipei, Taiwan) and visualized by Azure c400 system and AzureSpot analysis Software (Azure Biosystems, Dublin, CA, USA).

Immunofluorescence assay

HT-29 cells (1×10^5) were seeded in 6-cm dishes and cultured for 16–18 h. Cells were pretreated with simvastatin (20 μM) for 30 min followed by infection with *C. innocuum* at MOI of 100 for 4 h. Cells were fixed with 2% paraformaldehyde for 1 h and blocked with 3% skim milk for 1 h at room temperature. Cells were incubated with primary antibodies against NOD2 (GTX306615, dilution 1:400) overnight at 4°C, followed by Alexa FITC 488-conjugated goat anti-mouse IgG (dilution 1:1000, Invitrogen) and CTx-B Alexa Fluor 555-conjugate (dilution 1:200, Invitrogen) for 1 h at room temperature. Cells were stained with Hoechst 33,342 (dilution 1:1000) for 30 min at room temperature. The samples were observed under a confocal laser-scanning microscope (LSM780, Carl Zeiss, Germany) and the intensity of fluorescence was analysed by ZEN software (Carl Zeiss).

Cell viability assay

HT-29 cells (1×10^4) were seeded in 96-well plates and cultured for 16–18 h. Cells were respectively treated with *C. innocuum* (MOI = 500), *C. innocuum* culture broth, heat-treated *C. innocuum*, and cell lysate of *C. innocuum* for 24 h. Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)–2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, St. Louis, MO, USA). Cells were incubated with MTT reagent (0.5 mg/ml) at 37°C for 3 h. Isopropanol containing HCl was used to resolve the purple crystal for 30 min. The optical density at 570 nm was measured by SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Device, San Jose, CA, USA).

Analysis of apoptotic cells

HT-29 cells (2×10^6) were seeded in 6-cm dishes and cultured for 16–18 h. Cells were respectively treated with *C. innocuum* (MOI = 500), *C. innocuum* culture broth, heat-treated *C. innocuum*, and cell lysate of *C. innocuum* for 24 h. BHI broth was used as a negative control. The treated cells were collected and incubated with FITC-Annexin V in a buffer containing propidium iodide (PI) using the FITC Annexin V Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) according to the manufacturer's instructions. Fluorescence was analysed by FACS

Calibur System (BD Biosciences) by collecting 10,000 cells from each sample. All samples were performed of three independent experiments.

Luciferase activity assay

HT-29 cells (2×10^4) were seeded in 24-well plates and cultured for 16–18 h. pNF-κB-Luc (1 μg) and pGL3-Luc (1 μg) plasmids were co-transfected to cells by using jetPRIME (Polyplus-transfection, Illkirch-Graffenstaden, France) for 4 h. After transfection, cells were treated with simvastatin (100 μM) or treated with simvastatin then replenished with cholesterol (400 μg/ml), following by infection with *C. innocuum* (MOI = 500) for an additional 24 h. Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to analyse the luciferase reporter activity by following the manufacturer's instruction [20]. The luciferase activity was measured by SpectraMax iD3 Multi-Mode Microplate Reader (Molecular Device).

Analysis of IL-8 production

HT-29 cells (2×10^4) were seeded in 24-well plates and cultured for 16–18 h. Cells were treated with simvastatin (100 μM) or treated with simvastatin and replenished with cholesterol (400 μg/ml), following by infection with *C. innocuum* (MOI = 500) for an additional 24 h. The culture supernatant was collected and subjected to enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

All experiments were performed in triplicate repeats independently. Student's *t*-test was used to determine the statistical difference between two groups. The difference was considered significant when $P < 0.05$. Statistical analysis was conducted by using Prism Program (version 9.0.0 for Windows, GraphPad Software, La Jolla, CA, USA).

Results

C. innocuum induces NOD2 expression in colonic epithelial cells

Human colonic epithelial cells, HT-29, were used to determine NOD2 expression during *C. innocuum* infection. Our results showed that NOD2 and RIP2 expression levels in HT-29 cells were increased upon *C. innocuum* infection for 0–4 h (Figure 1a). The cells were then infected with *C. innocuum* at different MOIs (0–100). Both NOD2 and RIP2 levels were gradually elevated in cells infected with *C. innocuum* with an increased MOI (Figure 1b). These results suggest that NOD2 elevation may be involved in *C. innocuum*-induced inflammation of colonic epithelial cells.

Mobilization of NOD2 to lipid rafts in C. innocuum-infected cells

Next, we explored whether *C. innocuum* triggered NOD2 recruitment to membrane rafts. NOD2 was located on the cell membrane in the absence of *C. innocuum* (Figure 2). In cells infected with *C. innocuum*, NOD2 formed punctures at sites of bacterial attachment around the inner membrane and co-localized with CTX-B, which bound to ganglioside GM1 and served as a raft-marker. The merged image was then analysed using confocal microscopy *z*-section, which showed that the distribution of fluorescence intensity for *C. innocuum* and NOD2 was consistent with that of the raft-marker CTx-B across the cytoplasmic membrane (Figure 2d–h, right

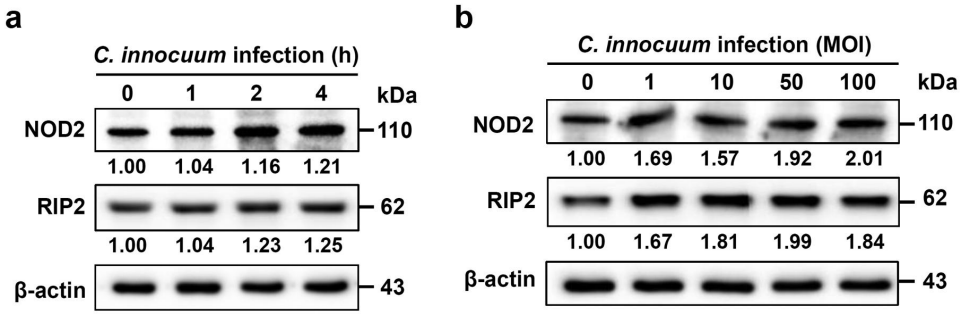

Figure 1. *C. innocuum* induces nucleotide-binding oligomerization domain containing 2 (NOD2) and receptor-interacting protein 2 (RIP2) expression. HT-29 cells were infected with *C. innocuum* at (A) multiplicity of infection (MOI) of 100 for 0–4 h, and (B) MOI of 0–100 for 4 h. Expression levels of NOD2 and RIP2 were determined using western blotting. The protein expression levels of NOD2 and RIP2 were quantified by densitometric analysis and normalized to β-actin level, respectively, and indicated at the bottom of each lane.

panels). These findings indicate that during *C. innocuum* infection, attached bacteria coalesce with NOD2, which is abundantly located in the membrane rafts.

Live C. innocuum *is essential for inducing cell death*

We recently reported that *C. innocuum* was cytotoxic to gut epithelial cells and caused severe pathogenic changes in mouse ileum [11]. Here, we further explored the mechanism by which *C. innocuum* induced cell death in human colonic epithelial cells. Live *C. innocuum*, bacterial culture supernatant, heat-killed cells, and bacterial lysates were examined for their cytotoxic activity in HT-29 cells. Our results showed that only live *C. innocuum* significantly decreased the cell viability, whereas other bacterial constituents did not influence the cell survival, as analysed by the MTT assay (Figure 3a). We then explored whether *C. innocuum*-induced cell death was mediated via the apoptotic pathway. Cells were treated with each bacterial preparation and stained with annexin V/PI, followed by flow cytometric analysis. Consistently, the proportion of apoptotic cells was remarkably increased in cells treated with live *C. innocuum* (Figure 3b–c). However, this trend was not observed in the bacterial supernatant or heat-killed bacteria. These results demonstrate that *C. innocuum*-induced cell death occurs via apoptosis and viable bacteria are required for this effect.

Reducing cholesterol levels ameliorates C. innocuum*-induced cell death*

We further investigated whether cholesterol was crucial for *C. innocuum*-induced pathogenesis. Simvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), was used to impede cellular cholesterol synthesis, and NOD2 expression on the cell membrane was analysed. Immunofluorescence staining showed that *C. innocuum* infection recruited a large proportion of NOD2 co-localized with CTx-B to the inner membrane compared to the bacterial uninfected group (Figure 4). In contrast, pretreatment of cells with simvastatin resulted in the reduction of NOD2 and CTx-B levels.

Effect of cholesterol inhibition on the cytotoxicity of HT-29 cells was then analysed. As shown in Figure 5a, pretreatment of cells with simvastatin, the cells were exposed to *C. innocuum* for 24 h, and the proportion of apoptotic cells was reduced. In contrast, the inhibitory effect of simvastatin on *C. innocuum*-induced apoptosis was abolished by cholesterol replenishment (Figure 5b). We then examined whether cholesterol is crucial for *C. innocuum*-induced inflammation in gut epithelial cells. Our results showed that *C. innocuum* infection remarkably increased NF-κB luciferase activity along with IL-8 production (Figure 5c,d). Consistently, after pretreatment of cells with simvastatin significantly decreased the *C. innocuum*-induced NF-κB

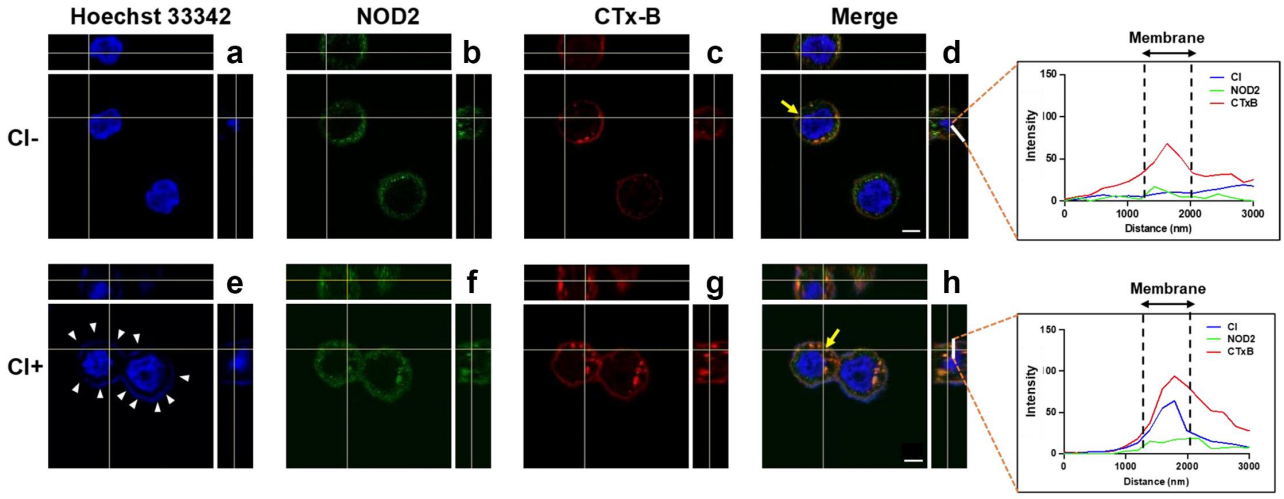

Figure 2. *C. innocuum* recruits NOD2 to membrane rafts. HT-29 cells were infected with *C. innocuum* at MOI of 100 for 4 h. Cells were probed with (A and E) Hoechst 33,342 (blue), (B and F) anti-NOD2 (green), and (C and G) CTx-B (red), followed by performing confocal microscopy analysis. (E) arrowheads indicate the attached bacteria on the cell membrane. (D and H) yellow arrows indicate the area of confocal *z*-section analysis. Bars, 5 μm. White lines across the membrane shown in *z*-section indicate the distribution of fluorescence signals for *C. innocuum* (blue line), NOD2 (green line), and CTx-B (red line), which present as line intensity histograms in the right panels.

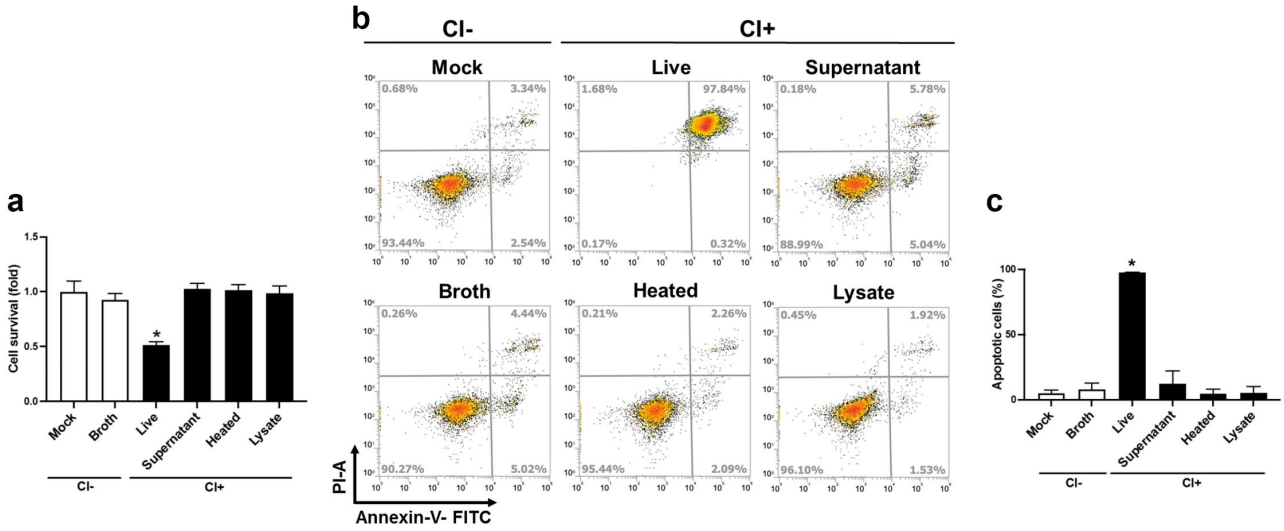

Figure 3. Live *C. innocuum* is essential for inducing cell death. HT-29 cells were treated with live *C. innocuum*, bacterial culture supernatant, heat-killed bacteria, and bacterial lysate for 24 h, followed by the (A) analysis of cell survival using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, (B) stained with annexin V/propidium iodide, and analyzed using flow cytometry. (C) percentage of apoptotic cells was quantitated. *, $P < 0.05$.

promoter activity and IL-8 production. These results demonstrate that sufficient cholesterol, present in lipid rafts, is essential for *C. innocuum*-associated pathogenesis.

Discussion

NOD2 can recognize MDP, which is mainly derived from the peptidoglycan of Gram-positive bacteria [12,21]. NOD2 activation promotes the recruitment of RIP2 to elicit the NF-κB and mitogen-activated protein kinase (MAPK) pathways [22], leading to the production of several cytokines/chemokines, such as interleukin (IL)-1β, IL-6, IL-8, and tumour necrosis factor (TNF)-α [23,24]. In contrast, *NOD2* mutations render it unresponsive to the bacterial cell wall component, MDP, revealing its important role in disease susceptibility and pathogenesis [25]. In line with previous studies, the current study demonstrated the impact of *C. innocuum* on the NOD2 pathway, which is crucial for the interaction of bacteria to trigger pathogenicity.

The present study showed that membrane-associated NOD2 was mobilized by *C. innocuum* to the inner membrane rather than the cell surface. These results are consistent with previous findings that NOD2 interacts with the inner side of the membrane rather than outer leaflet [26]. In addition, NOD2 recruits autophagy-related 16-like 1 (ATG16L1) to the cell membrane and facilitates the formation of autophagosomes around the invading bacteria [27]. Biochemical analyses showed that FERM and PDZ domain-containing 2 (FRMPD2) was selectively localized at the basolateral membrane in polarized epithelial cells interacting with the leucine-rich repeats of NOD2, indicating that FRMPD2 acts as a membrane anchor for NOD2 [28]. These findings indicate that membrane localization of NOD2 is not only important for bacterial entry, but also for maintaining the intestinal epithelial cell tolerance to commensal bacteria present at the apical side of the cells.

Membrane rafts are enriched in cholesterol and sphingolipids, which provide entry gates for bacteria and their toxin internalization, resulting in the deterioration of infectious diseases [29–31]. *C. difficile* transferase (CDT)-induced microtubule-based membrane protrusions rely on lipid rafts in human colon cells, indicating that CDT-action is lipid raft-dependent [32]. In addition, *C. difficile* SLP binds to membrane cholesterol microdomains and triggers inflammasome activation [17]. In the present study, we demonstrated that *C. innocuum* infection mobilizes NOD2 to lipid rafts, leading to the activation of NF-κB signalling, IL-8 production, and exacerbation of the cytotoxic effect on colonic epithelial cells. These results suggest that the virulence factors of *C. innocuum* are similar to those of *C. difficile* cell wall components (SLP and CwpV) [5,11,33], indicating that lipid rafts are essential for the action of virulence factors of both *C. innocuum* and *C. difficile*. Although two hypothetical proteins (CI_01447 and CI_01448) have been identified in *C. innocuum*, they are not cytotoxic to mammalian cells [34]. Different from *C. difficile*, the exact virulence factors that respond to *C. innocuum*-induced pathogenesis of colonic epithelial cells remain unknown.

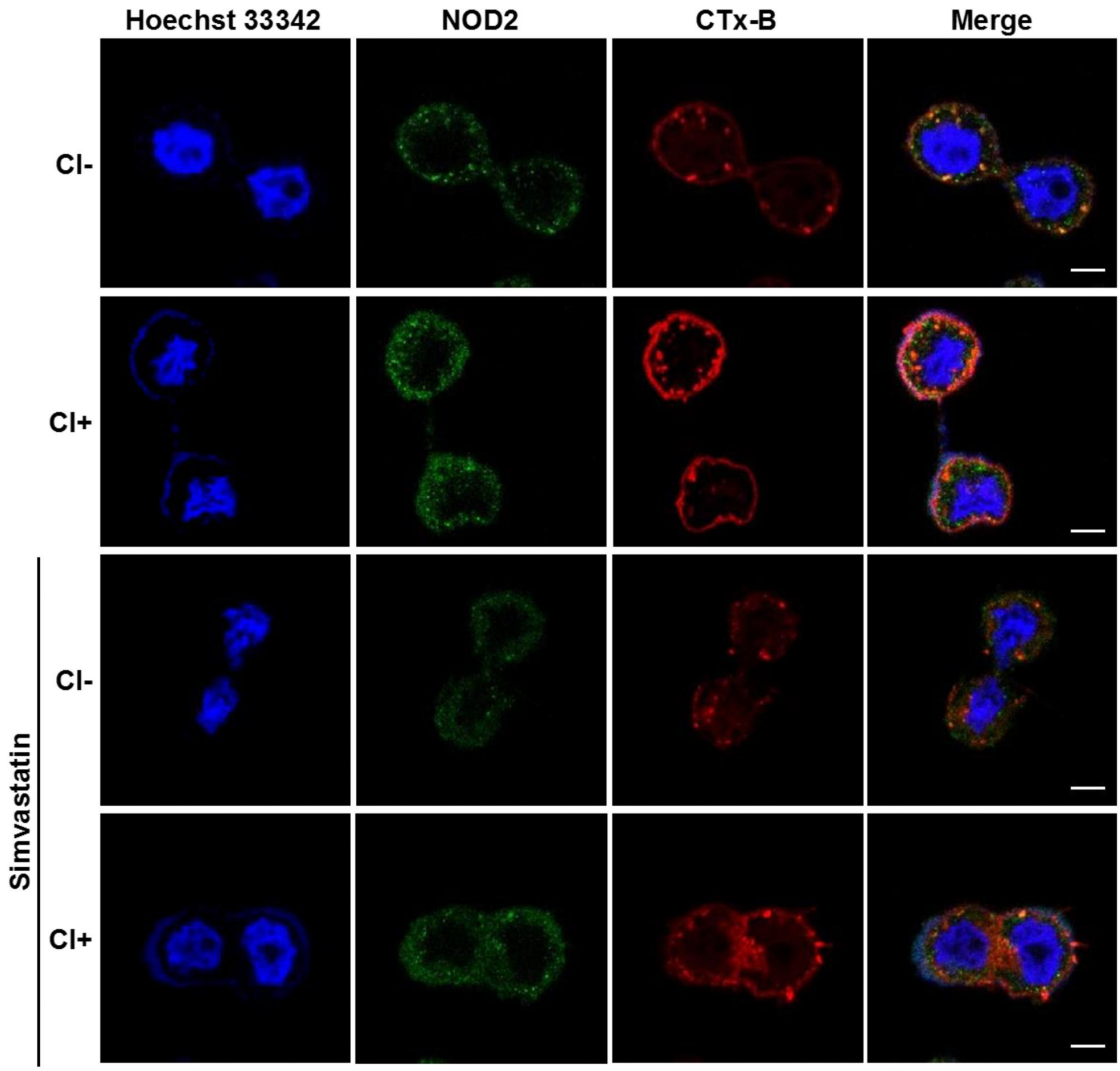

Figure 4. Inhibition of cholesterol synthesis reduces *C. innocuum*-induced NOD2 expression in lipid rafts. HT-29 cells were pretreated with simvastatin (100 μM) for 1 h prior to *C. innocuum* infection. Cells were probed with Hoechst 33,342 (blue), anti-NOD2 (green), and CTx-B (red), followed by confocal microscopy analysis. Bars, 5 μm.

Cholesterol usurping/depleting agents have been employed to ameliorate infectious agents by inhibiting their toxin-actions or preventing pathogen entry into host cells [35]. For example, statins, inhibitors of HMG-CoA reductase, effectively alleviate microbial infectivity [36–38]. Most importantly, statins can lower the cholesterol levels, which significantly decreases the risk [39] and improves the mortality of *C. difficile* infection [40,41] Consistently, a retrospective matched case-control study showed that statin prescription has a protective effect against new-onset IBD, including ulcerative colitis and Crohn's disease [42]. *C. innocuum* promotes the formation of creeping fat and prefers a lipid-rich environment [19,43]. However, to date, no studies have reported the association of *C. innocuum* with cholesterol and statin use for treating *C. innocuum*-related diseases. To the best of our knowledge, the present study is the first to reveal the essential role of cholesterol in the association of *C. innocuum* with membrane rafts, followed by the exacerbation of cytotoxicity. Our findings indicate that pharmaceutical agents that lower cholesterol levels can be developed to alleviate both *C. innocuum*- and *C. difficile*-related complications.

Owing to its intrinsic vancomycin-resistant nature, *C. innocuum* appears to be an opportunistic gut pathogen that causes gut dysbiosis [44]. *C. innocuum* co-infection with *C. difficile* commonly leads to a poorer clinical outcome than *C. difficile* infection alone in ulcerative colitis [7]. *C. innocuum* bacteraemia can be complicated by cytomegalovirus colitis [45]. In a patient with coronavirus disease 2019 (COVID-19), co-infection with cytomegalovirus and *C. innocuum* causes ulcer bleeding in the rectum [46]. Furthermore, patients with persistent respiratory symptoms are positively correlated with gut microbiota, including *C. innocuum* [47]. Together, these

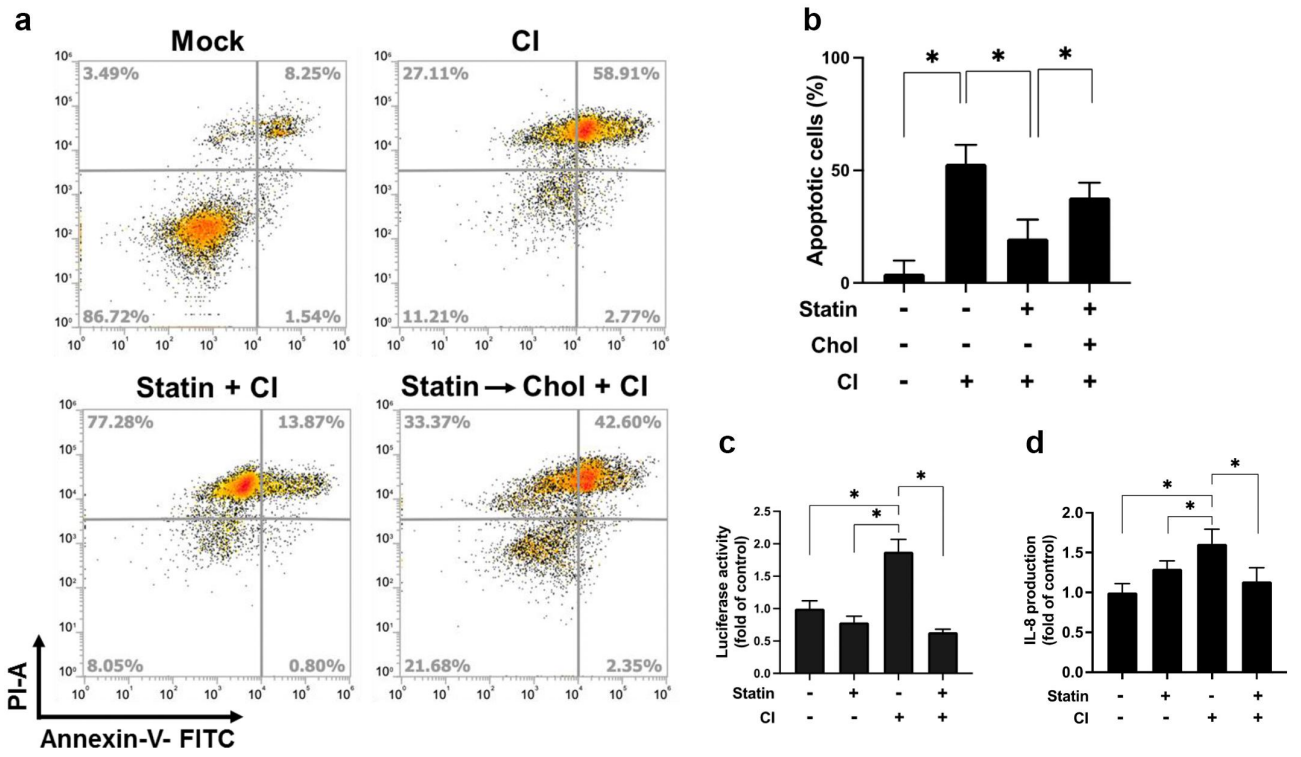

Figure 5. Simvastatin attenuates *C. innocuum*-induced apoptosis. (A) HT-29 cells were pretreated with simvastatin or pretreated with simvastatin then replenished with cholesterol (400 µg/mL). Cells were infected with *C. innocuum* for 24 h and stained with annexin V/propidium iodide, followed by flow cytometry analysis. (B) percentage of apoptotic cells was quantitated. (C) HT-29 cells were co-transfected with the nuclear factor (NF-κB) and pGL3 luciferase reporters and treated with simvastatin (100 µM) for 1 h, followed by *C. innocuum* infection for 24 h. NF-κB promoter activity was determined and normalized to pGL3 luciferase activity. (D) IL-8 production was analyzed by using ELISA. *, $P < 0.05$.

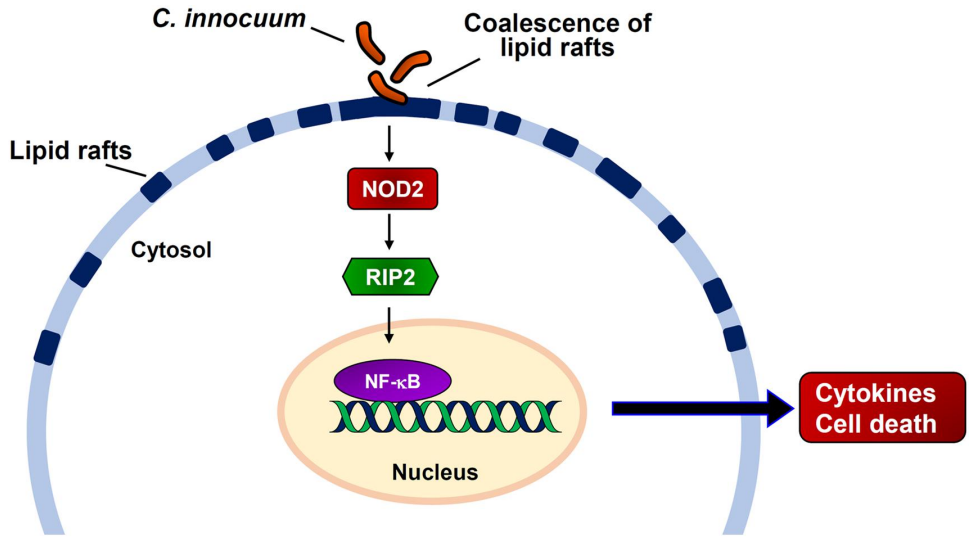

Figure 6. Hypothesized model illustrates *C. innocuum*-induced pathogenicity of intestinal epithelial cells. *C. innocuum* infection coalesces lipid rafts on the cell membrane, which then activates NOD2 pathway to promote NF-κB translocation in the nucleus, resulting in exacerbates cytotoxicity and inflammation of intestinal epithelial cells.

findings indicate that gut dysbiosis may provide a beneficial environment for *C. innocuum*, which has a survival advantage and ultimately causes various diseases. However, *C. innocuum* can be misidentified during co-infections with *C. innocuum* and *C. difficile* [48]. Virulence factors of *C. innocuum* responsible for its pathogenicity remain to be identified. In addition, the detailed mechanism of the interactions of *C. innocuum* with other gut pathogens that collaboratively exacerbate disease complications remains unclear. Moreover, precise approaches for identifying *C. innocuum* infections need to be explored in future studies.

In conclusion, our results showed that *C. innocuum* infection coalesced NOD2 to mobilize it in membrane rafts (Figure 6). We further demonstrated that *C. innocuum*-induced apoptosis of cells and viable bacteria were essential for the effect. Moreover, reducing cellular cholesterol levels using simvastatin decreased the NF-κB promoter activity, IL-8 production, and ameliorated *C. innocuum*-induced cytotoxicity, revealing that the *C. innocuum*-induced pathogenicity of cells is lipid raft-dependent. Unraveling the mechanism of *C. innocuum*-induced cytotoxicity in cells may contribute to the development of new strategies for controlling and treating *C. innocuum*-associated diseases.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author's contributions

Conception and design of this work: CCN, YMC, CHC, CHL
Experimental study: HYW, CJK, CHC, MWH, CLC, TSH, YCC
Data analysis and interpretation: HYW, CJK, CHC, MWH, CLC
Writing the manuscript: CHC, CHL
Final approval: all authors.

Data availability statement

The authors confirm that the data supporting the findings of this study are available within the article.

ORCID

Chih-Ho Lai http://orcid.org/0000-0001-7145-784X

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